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Homeoboxes in Sea Anemones (Cnidaria; Anthozoa): A PCR-Based Survey of *Nematostella vectensis* and *Metridium senile*

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Abstract. Homeobox genes belong to a phylogenetically widespread family of regulatory genes that play important roles in pattern formation and cell-fate specification in several model systems (*e.g., Drosophila,* mouse, and *C. elegans*). Although the evolution of many classes of homeobox genes predates the diversification of the Bilateria, comparatively little is known about homeobox genes in outgroups to the Bilateria, such as the Cnidaria. We used the polymerase chain reaction to recover 12 partial homeoboxes from 2 species of sea anemones, *Metridium senile* and *Nematostella vectensis* (phylum Cnidaria; class Anthozoa). These homeoboxes appear to represent 9 distinct, mutually paralogous homeobox genes, 5 of which belong to previously identified cnidarian homeobox classes, and 4 of which appear to represent previously unidentified classes. The evolutionary relationships between the homeodomains of sea anemones and of bilaterian animals were assessed through database searches and phylogenetic analyses. As many as 5 of the anemone homeoboxes may belong to the Hox class, which suggests that the Hox gene complement of cnidarians is larger than previously expected. Homologs of the *even-skipped* gene of *Drosophila* were also identified in both *Metridium* and *Nematostella.*

Introduction

Homeobox genes encode a family of transcription factors that are characterized by the presence of a DNA-binding domain consisting of 60 amino acids and known as the homeodomain. Homeobox genes have been found in all metazoan phyla that have been surveyed, as well as in plants and fungi (reviewed in Bürglin, 1994). Recent reports of homeobox genes from basal metazoans, such as sponges and cnidarians, are revising our understanding of the evolution of this multigene family (Murtha *et al.,* 1991; Schierwater *et al.,* 1991; Miles and Miller, 1992; Naito *et al.,* 1993; Kruse *et al.,* 1994, Seimiya *et al.,* 1994; Degnan *et al.,* 1995; Kuhn *et al.,* 1996). Research on homeobox genes has stimulated an ongoing synthesis between the fields of evolutionary biology and developmental genetics because of the possibility that homeobox genes have played an evolutionary role in the diversification of metazoan body plans.

We identified homeobox-containing genes in two species of sea anemone, *Metridium senile* and *Nematostella vectensis.* Sea anemones belong to the Cnidaria, a phylum of tentacle-bearing, radially symmetric animals that possess nematocysts (Hyman, 1940). Substantial phylogenetic evidence places the Cnidaria near the base of the Eumetazoa, possibly as the sister group to the Bilateria. The Cnidaria share with bilaterian animals several derived characters that distinguish eumetazoans from sponges, including the possession of epithelio-muscle cells and nerve cells. However, the cnidarian body plan lacks several derived features of the Bilateria: organ-level organization, a well-differentiated mesoderm, a coelom. Furthermore, cnidarians display radial or biradial symmetry instead of bilateral symmetry. The relative simplicity of the cnidarian body plan suggests that cnidarians may possess a network of developmental regulatory genes that is relatively simple and retains more of the primitive characteristics of early eumetazoan animals than do networks found among the bilaterians.

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Abbreviations: anth.hbx, anthozoan homeobox gene; *anthox,* anthozoan HOM/Hox class homeobox gene; *anth-eve,* anthozoan *even-skipped* class homeobox gene.

Therefore, study of cnidarians may provide unique insights into the evolution of developmental regulatory genes, such as homeobox genes. In any event, the Cnidaria are an outgroup to the Bilateria, so regardless of the degree of simplicity in their developmental regulatory networks, they will provide an important phylogenetic perspective on the evolution of development in the Metazoa.

Among extant Cnidaria, the Anthozoa (sea anemones and corals) are thought to be the sister group to a clade comprising the Cubozoa, Scyphozoa, and Hydrozoa, and it has been argued that the anthozoan body plan most resembles that of the ancestral cnidarians (Grasshoff, 1984; Schick, 1991; Bridge *et al.*, 1992; Odorico and Miller, 1997). Furthermore, the Anthozoa have a simpler life cycle than other Cnidaria, lacking the presumably derived medusoid stage that characterizes the Scyphozoa and the Hydrozoa. We have chosen to study homeobox genes in the Anthozoa that we might better understand the ancestral function of these genes in the Cnidaria and in the Eumetazoa.

Two techniques have been used to rapidly survey metazoan genomes for the presence of homeoboxes: (1) PCR (polymerase chain reaction) amplification of genomic DNA with degenerate primers corresponding to conserved regions of the homeodomain, and (2) library screening with a conserved oligonucleotide probe. More than 20 homeoboxes have been isolated from 8 cnidarian species by using these techniques (Murtha *et al.*, 1991; Schierwater *et al.*, 1991; Miles and Miller, 1992; Schummer *et al.*, 1992; Naito *et al.*, 1993; Shenk *et al.*, 1993; Aerne *et al.*, 1995). As many as five distinct homeoboxes have been recovered from individual hydrozoans, *Chlorohydra viridissima* (Schummer *et al.*, 1992) and *Eleutheria dichotoma* (Kuhn *et al.*, 1996). In a recent classification scheme (Naito *et al.*, 1993) cnidarian homeoboxes are sorted into eight mutually paralogous classes (Table I), but 18 of 20 of the cnidarian genes are from members of the class Hydrozoa. Little is known of homeobox genes in other classes, including the corals and anemones of the class Anthozoa. We have used PCR to survey the genome of two anthozoans for the presence of homeobox genes.

Materials and Methods

DNA extraction

Live anemones were obtained from William Zamer at Lake Forest College (Lake Forest, IL; *Metridium*) and Kevin Uhlinger at Bodega Bay Marine Laboratory (Bodega Bay, CA; *Nematostella*). All animals were starved for 2 weeks and rinsed several times in sterile artificial seawater prior to DNA extraction. DNA extraction was performed with proteinase K and RNase digestion followed by several phenol/chloroform extractions (Sambrook *et al.*, 1989).

PCR amplification

Degenerate primers corresponding to highly conserved regions in helices one and three of the homeodomain were used to amplify an 82-bp fragment from genomic DNA (nucleotides 60 through 141 of the homeobox, not including the primers). The upstream primer (5′-GARYTIGARAARGARTT-3′) corresponded to the amino acid sequence ELEKEF, and the downstream primer (5′-CKNCKRTTYTGRAACCA-3′) corresponded to the reverse complement for the amino acid sequence WFQNRR. The 5′ ends of the primers were phosphorylated to facilitate cloning PCR products. Both primers were synthesized commercially (Operon Technologies, Alameda, CA).

PCR was performed in 50-μl reactions containing 50 mM Tris (pH 8.3), 10 mM KCl, 3.5 mM $MgCl_2$, 200 μM dNTPs, 0.6 Units *Taq* polymerase, 12.5 pmol of each primer, and 150 ng genomic DNA. Thermal cycling was performed in a MiniCycler (MJ Research, Watertown, MA) under the following parameters: 5 cycles (45-s each) of denaturation at 94°C, 45 s of primer annealing at 37°C, and 45 s of primer extension at 72°C, followed by 35 cycles in which the annealing temperature was raised to 42°C. Eight separate 50-μl PCR reactions were performed for each species, and these were pooled prior to cloning to minimize the effects of stochastic variation known as PCR drift (Wagner *et al.*, 1994) Twenty-five microliters of the pooled reaction products were visualized on a 2% agarose gel stained with ethidium bromide, and the expected amplification product (114 bp) was observed. Control reactions lacking one primer of a primer pair failed to produce the expected amplification product. Control reactions lacking template produced no visible amplification products.

Cloning and sequencing

PCR products were blunted prior to cloning by using T4 DNA Polymerase (Costa and Weiner, 1994). The entire reaction was then run out on a 0.8% SeaPlaque GTG agarose gel (FMC Bioproducts, Rockland, ME) in a modified TAE buffer (0.04 M Tris-acetate, 0.2 mM EDTA). The appropriate band was excised from the gel, melted, and added directly to a ligation reaction. PCR products were cloned into pUC18 plasmid that had been cut with SmaI and treated with phosphatase (Kalvakolanu and Livingston, 1991). Following alkaline denaturation, double-stranded sequencing was performed on recombinant clones; the Sequenase v2.0 kit (USB, Cleveland, OH) was used with ^{35}S-labeled dATP and the M13 forward primer. Alternatively, automated sequencing was performed with dye-labeled dideoxynucleotides and

Table I

Previously identified homeoboxes from the phylum Cnidaria

Class[a] [putative homology]	Anthozoa[b,c] gene/[method of isolation]	Hydrozoa[b,c] gene/[method of isolation]
Class I [*HOM/Hox* class]		*cnox1*-Hm/[A][4] *cnox1*-Ed/[A][2] *SAnox3*-Sa/[A][1] *cnox1*-Hv/[A][5] *cnox3*-Hv/[A][5]
Class II [*Deformed* of *Drosophila*]		*cnox2*-Hv/[A][5] *cnox2*-Hm/[A][4] *cnox2*-Ed/[A][2] *cnox2*-Hs/[A][2] *cnox2*-Cv/[B][6] *SAnox2*-Sa/[A][1]
Class III [*Oct(1D), Drosophila*]		*cnox3*-Cv/[B][6] *cnox4*-Cv/[B][6]
Class IV [*HOM/Hox* class]		*cnox4*-Hm/[A][4] *cnox1*-Cv/[B][6]
Class V [*Mox*1 of mouse]		*cnox5*-Hm/[A][4]
Class VI [?]	*antpC*-Af/[B][3]	*SAnox1*-Sa/[A][1] *cnox1*-Pc/[A, B, C][7]
Class VII [*even-skipped* class]	*evcC*-Af/[B][3]	
Class VIII [*msh*-like class]		*msh*-Cv/[B][6]

[a] Naito *et al.* (1993) divided all previously identified cnidarian homeoboxes into eight classes. *Cnox1-Pc* and *antpC* were not part of the original classification scheme but have been assigned to Class VI based on phylogenetic analyses (see Figs. 2 and 3).

[b] All cnidarian homeobox sequences identified to date are from the Anthozoa and the Hydrozoa; the methods of isolation are indicated in brackets: [A] PCR, [B] genomic library screening, [C] cDNA library screening. No homeoboxes have yet been recovered from the classes Cubozoa or Scyphozoa. Two-letter species abbreviations are appended to the name of each gene: Af = *Acropora formosa*, Cv = *Chlorohydra viridissima*, Ed = *Eleutheria dichotoma*, Hm = *Hydra magnipapillata*, Hs = *Hydractinia symbiolongicarpus*, Hv = *Hydra vulgaris*, Pc = *Podocoryne carnea*, Sa = *Sarsia*.

[c] Homeoboxes listed here are from [1] Murtha *et al.* (1991), [2] Schierwater *et al.* (1991), [3] Miles and Miller (1992), [4] Naito *et al.* (1993), [5] Shenk *et al.* (1993), [6] Schummer *et al.* (1992), and [7] Aerne *et al.* (1995).

an ABI 373A sequencer. Homeobox-containing clones were sequenced in the reverse direction with the M13 reverse primer. Of the 44 clones sequenced for *Metridium*, 32 (73%) contained recognizable homeobox sequences. Of the 47 clones sequenced for *Nematostella*, 35 (74%) contained recognizable homeobox sequences.

Results and Discussion

Homeoboxes identified by the PCR-based survey

Our PCR-based survey identified 12 distinct homeoboxes in two species of sea anemone: 7 genes from *Metridium senile* and 5 from *Nematostella vectensis* (Fig. 1). Consensus sequences for each of these 12 anemone homeobox gene fragments were submitted to GenBank (accession numbers U42726-U42737). To our knowledge, the 7 homeoboxes identified in *Metridium* are the most recovered from any single species of cnidarian. The recovery of so many distinct genes may be attributable to the initial pooling of 8 separate PCR reactions (Wagner *et al.*, 1994). The 12 anemone homeoboxes appear to represent 9 paralogous homeobox genes referred to as *anthox1*, *anthox1a*, *anthox2*, *anthox4*, *anthox5*, *anth-hbxA*, *anth-hbxB*, *anth-hbxC*, and *anth-eve*. The names were chosen to reflect putative homology between anemone homeoboxes and those from other cnidarians (see legend to Fig. 1). The PCR surveys identified overlapping but different sets of homeoboxes in *Metridium* and *Nematostella*. *Anthox1*, *anth-eve*, and *anth-hbxA* were recovered from both *Metridium* and *Nematostella*. *Anthox2*, *anthox4*, *anthox5*, and *anth-hbxB* were recov-

Figure 1. Nucleotide alignments of homeobox-containing clones representing 12 distinct homeobox genes, 7 from *Metridium senile* and 5 from *Nematostella vectensis*. The nucleotide alignment spans positions 61–142 of the 180-bp homeobox (amino acids 21–47 of the homeodomain). Nucleotide alignments and phylogenetic analysis suggest that these 12 homeobox fragments represent at least 9 distinct homeobox classes or cognate groups (in boldface type). The term *anthox* is used to designate anemone homeoboxes with putative homology to Hox class homeoboxes (anthozoan Hox homeobox). The term *anth-hbx* designates anemone homeoboxes lacking similarity to the Hox class. The term *anth-eve* indicates the apparent homology of this anemone homeobox fragment to the *even-skipped* homeobox of *Drosophila*. The sequences of individual clones (lowercase) are aligned to a consensus sequence (uppercase). For *anthox1, anthox2, anthox4, anthox5, anth.hbxA, anth.hbxB,* and *anth-eve*, the consensus sequence shown is from *Metridium*. For *anth.hbxC* and *anthox1a* the consensus sequence shown is from *Nematostella*. The inferred amino acid sequences are indicated below the consensus. The number of clones possessing a given sequence is indicated in parentheses. Identity with the consensus sequence is indicated with a period.

ered only in *Metridium*. *Anthox1a* and *anth-hbxC* were recovered only in *Nematostella*.

Comparison of homeoboxes from anemones and other cnidarians

We wanted to know if the homeoboxes we isolated are orthologous to previously identified homeoboxes from cnidarians (Table I; Naito *et al.*, 1993), or if they represent new classes of cnidarian homeoboxes. We used phylogenetic methods to evaluate the evolutionary relationships between representatives of the 8 putative cnidarian classes and the 12 homeoboxes recovered from *Metridium* and *Nematostella* (Saitou and Nei, 1987; Fig. 2). A neighbor-joining tree (Saitou and Nei, 1987) was constructed using the program *Phylip* (version 3.5,

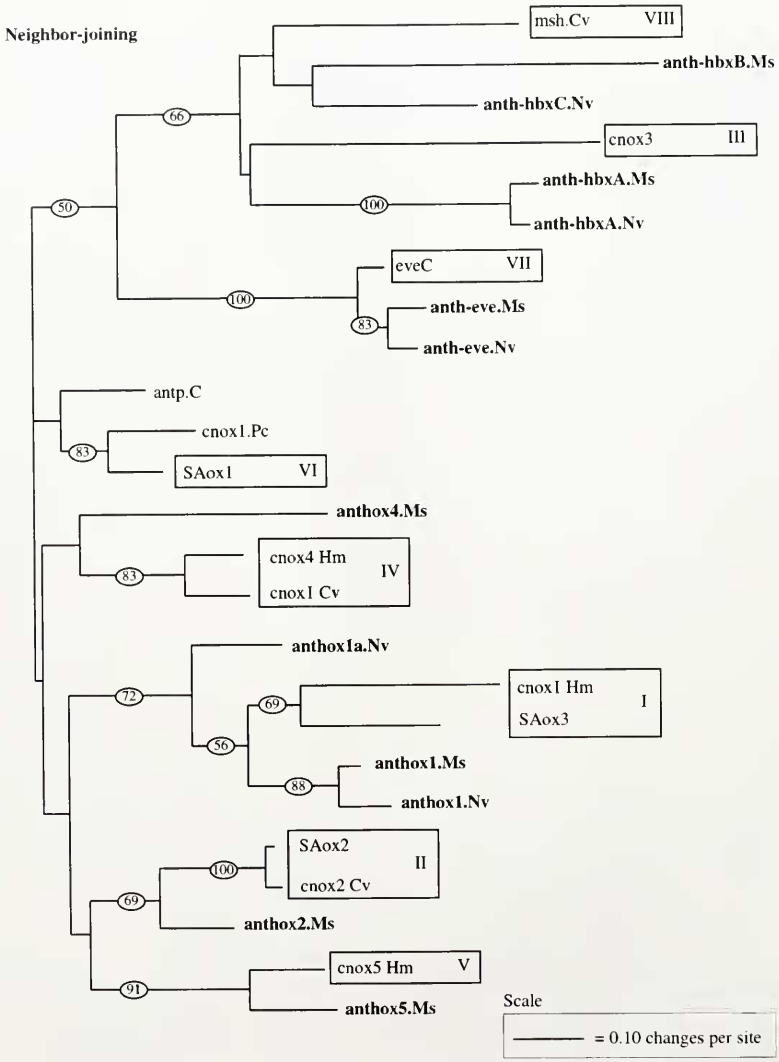

Figure 2. Phylogenetic relationships among cnidarian homeodomains were inferred using the neighbor-joining algorithm (Saitou and Nei, 1987) of PHYLIP (Phylogenetic Inference Package, version 3.5; Felsenstein, 1989). The data consisted of predicted amino acid sequences (positions 21–47; see Fig. 1) from the 12 anemone homeodomains and 13 additional cnidarian homeodomains. Anemone homeodomains are indicated in boldface type. The cnidarian sequences were chosen to represent all 8 of the cnidarian homeobox classes proposed by Naito *et al.* (1993). Boxes enclose representative sequences from each class (roman numerals; see Table I). Sequences not enclosed in boxes (*cnox1 Pc* and *antpC*) were not included in the classification scheme of Naito *et al.* (1993). The tree shown is unrooted. Distances between homeodomains were calculated using the Protdist program of PHYLIP and the PAM-Dayhoff amino acid substitution matrix (Dayhoff, 1978). Branch lengths in the horizontal direction are proportional to the number of expected amino acid substitutions that have occurred per site (scale at lower right). Branch lengths in the vertical direction are not significant. Circled numbers along branches indicate the percentage of trials in which a given partition between genes was found in 2000 replications of the bootstrap resampling procedure (Felsenstein, 1985). Only partitions supported in >40% of bootstrap replicates are indicated.

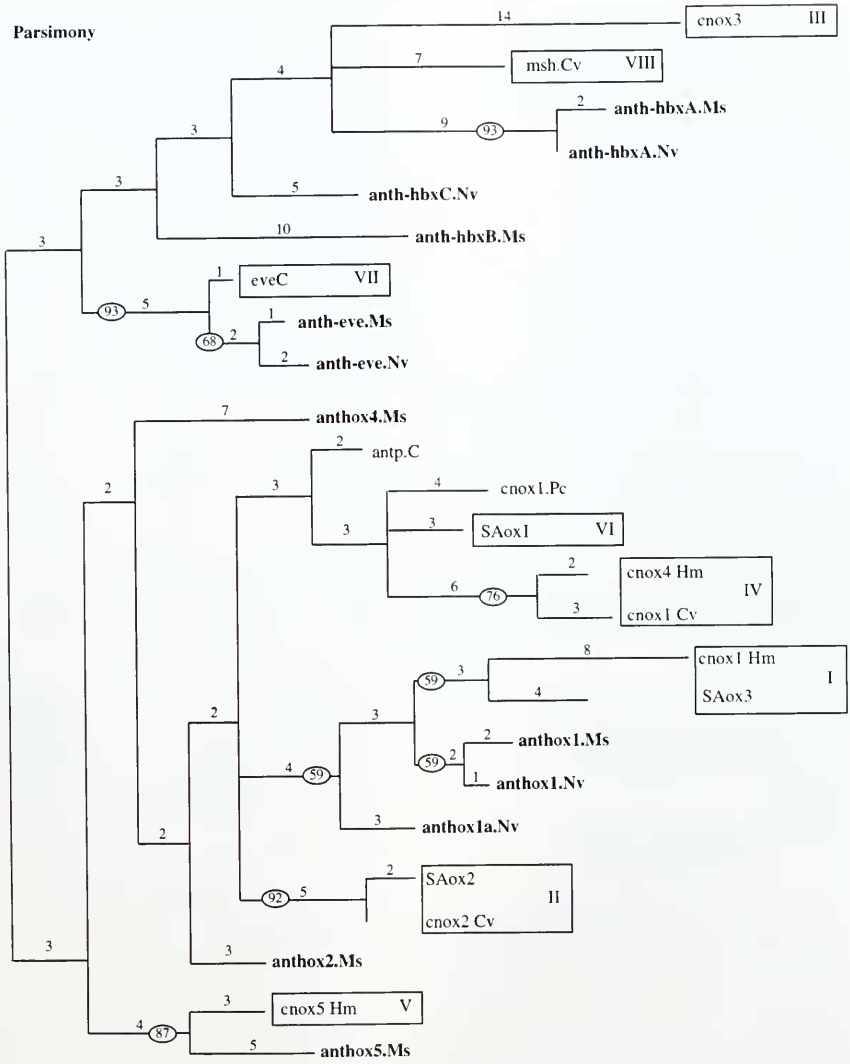

Figure 3. Phylogenetic relationships among cnidarian homeodomains were inferred by parsimony. The tree depicted is the strict consensus of 27 equally parsimonious trees identified in a heuristic search with the computer program *PAUP* (*Phylogenetic Analysis Using Parsimony*, version 3.1, Swofford, 1991). Tree-bisection and reconnection branch swapping (TBR) was performed on 20 random starting trees. The tree shown is unrooted. The data consisted of predicted amino acid sequences (positions 21–47; see Fig. 1) from the 12 anemone homeodomains and 13 additional cnidarian homeodomains representing 8 distinct classes (Naito *et al.*, 1993; Table I). Anemone homeodomains are indicated in boldface type. Boxes enclose representatives from each class (roman numerals). Sequences not enclosed in boxes (*cnox1.Pc* and *antpC*) were not included in the classification scheme of Naito *et al.* (1993). Tree length is 162 steps. Consistency index (CI) = 0.691. Retention index (RI) = 0.695. Branch lengths in the horizontal direction are proportional to the number of amino acid substitutions that have occurred along each branch. The number of amino acid substitutions is specified above each branch. Branch lengths in the vertical direction are not significant. Circled numbers indicate the percentage of trials in which a given partition between genes was found in 500 replications of the bootstrap resampling procedure (Felsenstein, 1985). Only partitions supported in >50% of bootstrap replicates are indicated.

		% identity
anthox1.Ms	HFNHFLTKERRSEMATQLNLTDRQVKI	
1. CnHv3 (*Hydra vulgaris*) [M62872]	?.....K....A.L.KH...SE..I.?	68%
2. Abd-A homolog (*Aedes aegypti*) [P29552]	....Y..RR..I.I.HA.C..E..I..	68%
3. Hox D3 (*Homo sapiens*) [S15548]	...RY.CRP..V...NL....E..I..	62%
4. CnHv2 (*Hydra vulgaris*) [M62871]	?.....K....T.LSKK...SE..I.?	64%
5. Hox 3 (*Branchiostoma floridae*) [D44629]	...RY.CRP..V...AM....E..I..	64%
anthox1.Nv	HFNHFLTKERRSEMRSQLNLTERQVKI	
1. CnHv2 (*Hydra vulgaris*) [M62871]	?.....K....T.LSKK...S...I.?	68%
2. CnHv3 (*Hydra vulgaris*) [M62872]	?.....K....A.LAKH...S...I.?	63%
3. Abd-A homolog (*Aedes aegypti*) [P29552]	....Y..RR..I.IAHA.C.....I..	63%
4. Hox D3 (*Homo sapiens*) [S15548]	...RY.CRP..V..ANL.......I..	63%
5. Hox 3 (*Branchiostoma floridae*) [D44629]	...RY.CRP..V..AAM.......I..	63%
anthox1a.Nv	HFTRYLTKERRTEMARMLNLTERQVKI	
1. Hox A3 (Mus musculus) [P02831]	..N...MRP..V...NL.......I..	70%
2. Hox D3 (*Homo sapiens*) [S15548]	..N...CRP..V...NL.......I..	70%
3. Hox 3 (*Branchiostoma floridae*) [D44629]	..N...CRP..V...A........I..	74%
4. Hox B3 (*Homo sapiens*) [D37042]	..N...CRP..V...NL...S...I..	67%
5. Hox B3 (Xenopus laevis) [P31247]	..N...CRP..V...NL...S...I..	67%
anthox2.Ms	HYNRYLCRPRRIEIAQSLDLTEKQVKI	
1. Homeobox (*Carassius auratus*) [L09690]	...K.......V...AL.....R...V	78%
2. Hox A2 (*Notophthalmus viridescens*) [P31261]	.F.K.......V...AL.....R...V	74%
3. Hox B2 (*Homo sapiens*) [E37042]	.F.K.......V...AL.....R...V	74%
4. Hox B2 (*Mus musculus*) [A35511]	.F.K.......V...AL.....R...V	74%
5. Hox 2 (*Saccoglossus kowalevskii*) [C44636]	...K............SM...S.R...V	78%
anthox4.Ms	LYSRYLTRTRRLELAKSLGLSEKHLKI	
1. Hox C4 (*Rattus norvegicus*) [P18865]	H.N.....R..I.I.H..C...RQI..	63%
2. Hox 8 (*Branchistoma floridae*) [I44629]	HFN.....R..I.I.H....T.RQI..	59%
3. Hox 5 (*Saccoglossus kowalevskii*) [F44636]	HFN.....R..I.I.HA.....RQI..	59%
4. Hox 4 (*Petromyzon marinus*) [H48200]	.FN.....R..L.I.H..A...RQV..	63%
5. HB3 (*Tripneustes gratilla*) [P10178]	HF...V..R..F.I.Q......RQI..	63%
anthox5.Ms	TKNNYLTRLRRYEIAMGLDLAERQVKV	
1. Cnox5 (*Hydra magnipapillata*) [S39068]	VR.............VS.S.S.....	78%
2. mox-2 (*Mus musculus*) [S29902]	AHH............VN...T.....	78%
3. homeotic protein (*Dugesia tigrina*) [S32729]	AVH..........L.VA.N.N...I..	67%
4. btn gene product (*Drosophila*) [S75228]	CYS............VA.E.T.....	74%
5. mox-1 (*Mus musculus*) [P32442]	AHH............VN...S.....	78%
anth-eve.Ms	LRENYVSRTRRCELANSLSLSETTIKI	
1. eveC (*Acropora formosa*) [S36770]	..........S...SM.N........	85%
2. Xhox-3 (*Xenopus laevis*) [A60092]	Y.......P......AA.N.P.....V	74%
3. eve (*Drosophila melanogaster*) [X05138]	YK......P......AQ.N.P.S...V	67%
4. EVX2 (*Homo sapiens*) [M59983]	Y.......P......AA.N.P.....V	74%
5. EVX1 (*Homo sapiens*) [S22586]	Y.......P......AA.N.P.....V	74%
anth-eve.Nv	MRENYVSRTRRCELANALNLSETTIKI	
1. eveC (*Acropora formosa*) [S36770]	L..........S...SM..........	85%
2. Xhox-3 (*Xenopus laevis*) [A60092]	Y.......P......AA...P.....V	78%
3. EVX2 (*Homo sapiens*) [M59983]	Y.......P......AA...P.....V	78%
4. eve (*Drosophila melanogaster*) [X05138]	YK......P......AQ...P.S...V	70%
5. EVX1 (*Homo sapiens*) [S22586]	Y.......P......AA...P.....V	78%
anth-hbxA.Ms	GEEKYLTEAKRAELSKDLGMTETQVKT	
1. EgHbx2 (*Echinococcus granulosus*) [JC1387]	DRQ...SS.E...M.R...LS.....I	59%
2. homeo A-1 (*Echinostoma trivolvis*) [L19170]	HFNR...R.R.I.IAN...L......I	56%
3. engrailed (*Drosophila melanogaster*) [K03059]	N.NR....RR.Q...SE..LN.A.I.I	52%
4. homeobox (*Heliocidaris erythrogramma*) [U20895]	KAD...SVS..L...MS.NL....I..	56%
5. E30 (*Apis mellifera*) [P09076]	A.NR....RR.QQ..R...L..A.I.I	56%
anth-hbxA.Nv	KEEKYLTESKRAELSKDLGMTETQVKT	
1. EgHbx2 (*Echinococcus granulosus*) [JC1387]	DRQ...SSAE...M.R...LS.....I	56%
2. homeobox (*Heliocidaris erythrogramma*) [U20895]	.AD...SV...L...MS.NL....I..	63%
3. E30 (*Apis mellifera*) [P09076]	A.NR....RR.QQ..R...L..A.I.I	56%
4. engrailed (*Drosophila melanogaster*) [K03059]	N.NR....RR.Q...SE..LN.A.I.I	52%
2. homeo A-1 (*Echinostoma trivolvis*) [L19170]	HFNR...RAR.I.IAN...L......?	52%
anth-hbxB.Ms	ERQQYMVGAERHYLAASLNLTETQVKV	
1. EMX2 (*Mus musculus*) [Q04744]	.KNH.V.....KQ..H..S........	70%
2. EMX1 (*Mus musculus*) [Q04742]	.KNH.V.....KQ..G..S.S......	67%
3. Cnot 1 (*Gallus gallus*) [X82575]	LK......T..VD...T.R........	74%
4. Xnot 1(*Xenopus laevis*) [A46305]	LK......T..VD..ST..........	74%
5. EMX2 (*Homo sapiens*) [Q04743]	.KNH.V.....KQ..H..S........	70%
anth-hbxC.Nv	EAKKYLTATERSDMASLLNVTETQVKI	
1. Hox A8 (*Branchiostoma floridae*) [L14867]	.V....S.S..A.L.A.....D...RK	67%
2. ceh-9 (*Caenorhabditis elegans*) [S13129]	......SSSD..EL.KR.D........	67%
3. msx-2 (*Mus musculus*) [PS0410]	RQ.Q..SIA..AEFS.S..L........	56%
4. H17 (*Apis mellifera*) [P15857]	RE.Q...IA..AEFS.S.HL........	56%
5. msx-2 (*Gallus gallus*) [H45187]	RQ.Q..SIA..AEFS.S..L........	56%

Felsenstein, 1989; Fig. 2), and a parsimony tree was constructed using the program *PAUP (Phylogenetic Analysis Using Parsimony*, version 3.1; Swofford, 1991; Fig. 3).

On the gene phylogenies (Figs. 2 and 3), Class I, Class II, and Class IV, as defined by Naito *et al.* (1993; Table I) are monophyletic. Among the anemone homeodomains, *anthox1* of *Metridium* seems to be orthologous to *anthox1* of *Nematostella*. *Anth-eve* of *Metridium* and *Nematostella* also appear orthologous, as do *anth-hbxA* of *Metridium* and *Nematostella*. The phylogenies also permit a tentative assignment of some of the anemone homeodomains to specific cnidarian classes. *Anth-eve* appears orthologous to *eveCoral* (Class VII). *Anthox1* and *anthox1a* cluster with members of cnidarian Class I that are believed to be related to the *Antennapedia*-like, or Hox class, homeoboxes (Naito *et al.*, 1993). *Anthox5* appears orthologous to *cnox5* (Class V). Other associations appear more tenuous.

Identity of anemone homeoboxes assessed by searching the Genbank database

The sequences of anemone homeodomains inferred from the gene fragments recovered in this study were compared to sequences in GenBank with the BLASTx network search algorithm (Basic Local Alignment Search Tool; Altschul *et al.*, 1990). The top five matches recovered in the BLAST searches are aligned to their anemone homeodomain counterparts in Figure 4. For each of the unique anemone homeodomain fragments, the search was limited to the 50 most similar genes in the GenBank database. In every case, all 50 of the genes identified were homeobox genes. At the amino acid level, anemone homeodomains are 59%–85% identical to the most similar homeobox genes in the GenBank database. The BLAST searches reveal that five of the anemone sequences are most similar to homeodomains of the Hox class: these are *anthox1*, *anthox1a*, *anthox2*, *anthox4*, and *anthox5*. The *anth-eve* homeodomains are most similar to *even-skipped* homologs from other species, particularly the *even-skipped* homolog of the staghorn coral (Miles and Miller, 1992). The *anth-hbxA* homeodomains from both *Metridium* and *Nematostella* appear most similar to the flatworm homeodomain *EgHbx2* (Oliver *et al.*, 1992). *EgHbx2* has been referred to as an *NK* type homeobox, but this assignment is not supported by a recent homeobox gene tree (Bürglin, 1994). The *anth-hbxA* homeodomains also resemble the *engrailed* homeodomain of *Drosophila*. *Anth-hbxB* of *Metridium* is most similar to *empty-spiracles* homologs from the mouse (EMX1 and EMX2). *Anth-hbxC* from *Nematostella* exhibits similarity to a diverse group of genes, including Hox A8 from *Branchiostoma* and *msx2* from mouse, two members of the *msh*-class of homeodomains.

Phylogenetic relationships of homeoboxes from sea anemones and higher metazoans

Phylogenetic analyses of cnidarians and bilaterians were used to further assess the identity of the anemone homeoboxes (Saitou and Nei, 1987). In addition to the 12 anemone homeodomains and 13 sequences from other cnidarians, our analysis included 20 sequences from bilaterian animals, each representing a different class of homeodomains (Bürglin, 1994). A neighbor-joining tree (Fig. 5) supports the finding that some of the anemone homeoboxes belong to previously proposed cnidarian classes (Naito *et al.*, 1993; see previous section *Comparison of homeoboxes from anemones and other cnidarians*). *Anthox1* allies with members of Class I (*cnox1.Hm* and *SAox3*), *anthox2* with members of Class II (*cnox2.Cv* and *SAox2*), *anthox4* with members of Class IV (*cnox4.Hm* and *cnox1.Cv*), *anthox5* with Class V (*cnox5.Hm*), and *anth-eve* with Class VII (*eveC*). All of these findings, with the exception of the precise relationships of *anthox4*, are supported by a parsimony analysis (Fig. 6).

The phylogenetic analyses suggest that certain anemone homeoboxes represent classes previously unidentified in the Cnidaria (Figs. 5 and 6). *Anthox1a* from *Nematostella* appears as the sister group to the Class I genes and may represent a new class of cnidarian homeoboxes (Class Ia). *Anth-hbxA*, found in both *Metridium* and *Nematostella*, appears most closely related to the homeobox from the human gene TCL-3, a non-cluster member of the Hexapeptide superclass (Bürglin, 1994). As suggested by the BLASTx search results (Fig. 3), *Anth-hbxB* from *Metridium* appears most closely related to the *Drosophila empty-spiracles* homeobox, a gene known to be involved in head development in both *Drosophila* and vertebrates (Walldorf and Gehring, 1992; Simeone *et al.*, 1992). Finally, *Anth-hbxC* appears most closely related to the *C. elegans* homeobox *ceh-9*. The PCR surveys of sea anemones failed to recover representatives of cnidarian classes 3, 4, 6, or 8. The BLAST searches and phy-

Figure 4. Alignments of anemone homeodomain fragments to similar homeodomains identified by searching the GenBank molecular database with the BLASTx algorithm (*Basic Local Alignment Search Tool*; Altschul *et al.*, 1990). The predicted amino acid sequences of anemone homeodomains (amino acids 21–47) are aligned to the five best matches identified by the database search in order of decreasing similarity to the query sequence. Periods indicate identity with the anemone homeodomain. Accession numbers of GenBank sequences are presented in brackets.

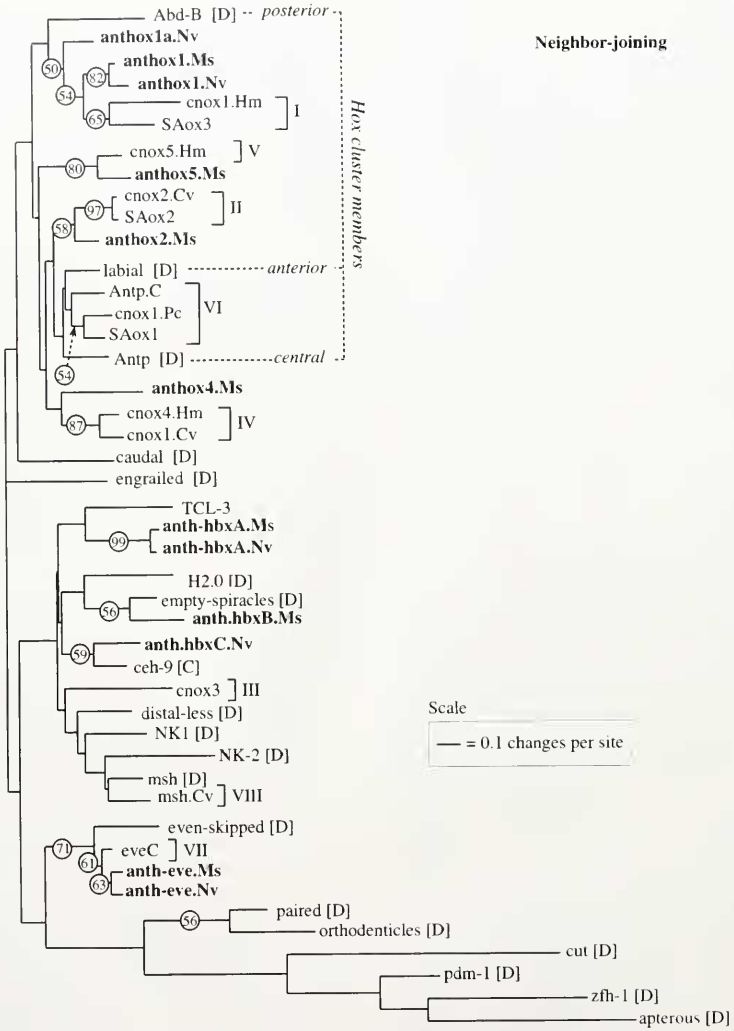

Figure 5. Phylogenetic relationships of predicted homeodomain sequences from cnidarians and bilaterians were inferred using the neighbor-joining algorithm (Saitou and Nei, 1987). The tree was generated as described in Figure 2. Cnidarian homeodomain sequences are labeled as in Figures 2 and 3, except that brackets are used to identify representative sequences from each cnidarian class (Naito *et al.*, 1993; roman numerals). Non-cnidarian homeodomain sequences were chosen to represent each of the 20 proposed classes of homeobox genes that make up an existing classification scheme (Bürglin, 1994). Non-cnidarian sequences are from *Drosophila* [D], *C. elegans* [C], and *Homo sapiens* [H]. Branch lengths in the horizontal direction are proportional to the number of expected amino acid substitutions that have occurred per site (scale at lower right). Branch lengths in the vertical direction are not significant. Circled numbers along branches indicate the percentage of trials in which a given partition between genes was found in 2000 replications of the bootstrap resampling procedure (Felsenstein, 1985). Only partitions supported in >40% of bootstrap replicates are indicated. Dashed lines indicate homeoboxes of the Hox class from *Drosophila*. These genes have differing anterior borders of expression along the anterior-posterior axis of the developing embryo. *Labial* is expressed more anteriorly than *Antennapedia* (Antp), which in turn is expressed more anteriorly than *AbdominalB* (AbdB). For this reason, Hox genes are sometimes classified according to their expression pattern in the embryo as anterior (*e.g., labial*), central (*e.g., Antennapedia*), and posterior (*e.g., AbdominalB*).

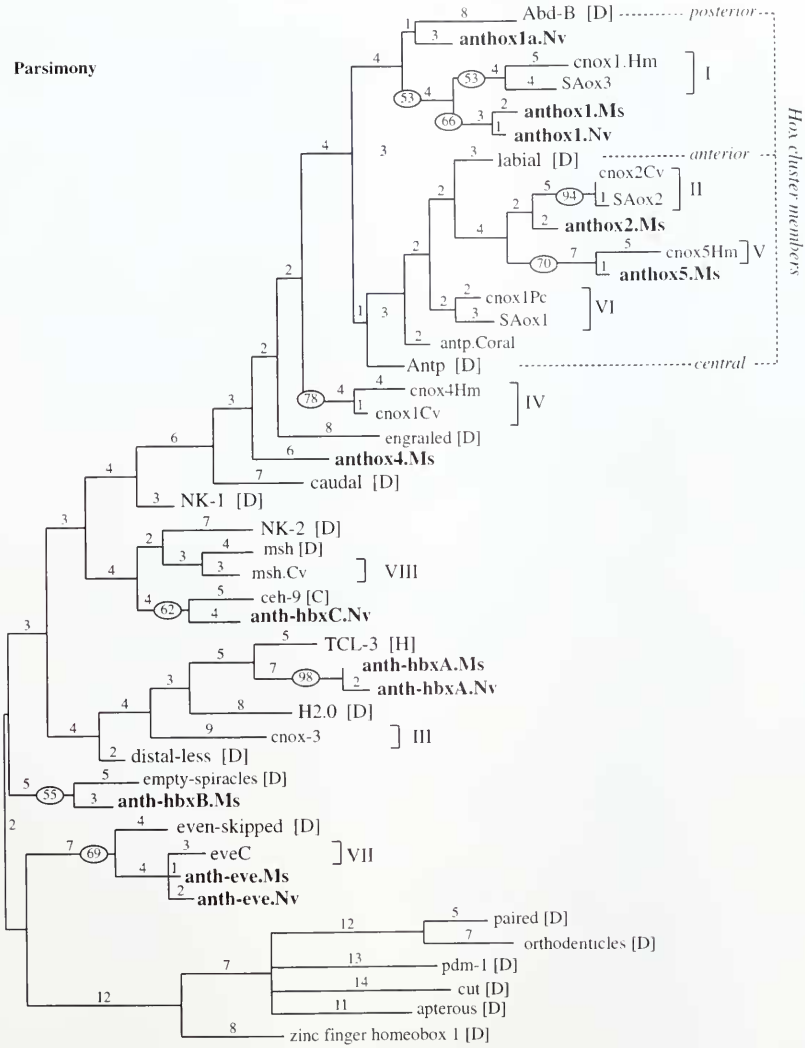

Figure 6. Phylogenetic relationships of predicted homeodomain sequences from cnidarians and bilaterians were inferred using the maximum parsimony criterion. The tree was generated as in Figure 3. Sequences used are identical to those in Figure 5. Anemone homeodomains are indicated in boldface type. The tree depicted is a 50% majority-rule consensus of 123 equally parsimonious trees identified by tree-bisection and reconnection (TBR) branch swapping on 20 random starting trees. Tree length = 354 steps. Consistency index (CI) = 0.506. Retention index (RI) = 0.557. Branch lengths in the horizontal direction are proportional to the number of amino acid substitutions that have occurred along each branch. The number of amino acid substitutions is specified above each branch. Branch lengths in the vertical direction are not significant. Circled numbers indicate the percentage of trials in which a given partition between genes was found in 200 replications of the bootstrap resampling procedure (Felsenstein, 1985). Only partitions supported in >50% of bootstrap replicates are indicated.

Table II

Proposed classification of cnidarian homeoboxes based on BLASTx searches and phylogenetic analysis

	Homeobox Class											
Cnidarian Bilaterian†	I Hox	Ia Hox	II Hox	III dll/S59	IV Hox	V Hox	VI Hox	VII even-skipped	VIII msh	IX TCL-3	X empty-spiracles	XI ceh-9
Species												
Class Hydrozoa												
Chlorohydra viridissima			*cnox2*	*cnox3*	*cnox1*				*msh.Cv*			
Eleutheria dichotoma	*cnox1*		*cnox2**									
Hydra magnipapillata	*cnox1*		*cnox2**		*cnox4*	*cnox5*						
Hydra vulgaris			*cnox2**									
Hydractinia symbiolongicarpus			*cnox2**									
Podocoryne carnea							*cnox1*					
Sarsia	*SAox3*		*SAox2*				*SAox1*					
Class Anthozoa												
Metridium senile	*anthox1*		*anthox2*		*anthox4***	*anthox5*		*anth-eve*		*anth-hbxA*	*anth-hbxB*	
Nematostella vectensis	*anthox1*	*anthox1a*						*anth-eve*		*anth-hbxA*		*anth-hbxC*
Acorpora formosa							*antpC*	*eveC*				

This classification scheme for cnidarian homeoboxes builds on that suggested by Naito *et al.* (1993) and is consistent with the results of BLAST searches (Fig. 4) and phylogenetic analyses (Figs. 5 and 6).

† The bilaterian classification scheme is taken from Bürglin (1994). Homology between cnidarian and bilaterian classes is based on the phylogenetic analyses (Figs. 5 and 6). New cnidarian classes suggested by the sea anemone data are Classes Ia, IX, X, and XI.

* Cnox2 from *Eleutheria dichotoma*, *Hydra magnipapillata*, *Hydra vulgaris*, and *Hydractinia symbiolongicarpus* was not included in the phylogenetic analyses because the homeodomains have predicted amino acid sequence identical to that of cnox2 from *Chlorohydra viridissima*

** The neighbor-joining tree (Fig. 5) supports inclusion of anthox4 in class IV, whereas the parsimony tree (Fig. 6) suggests that anthox4 represents a novel class.

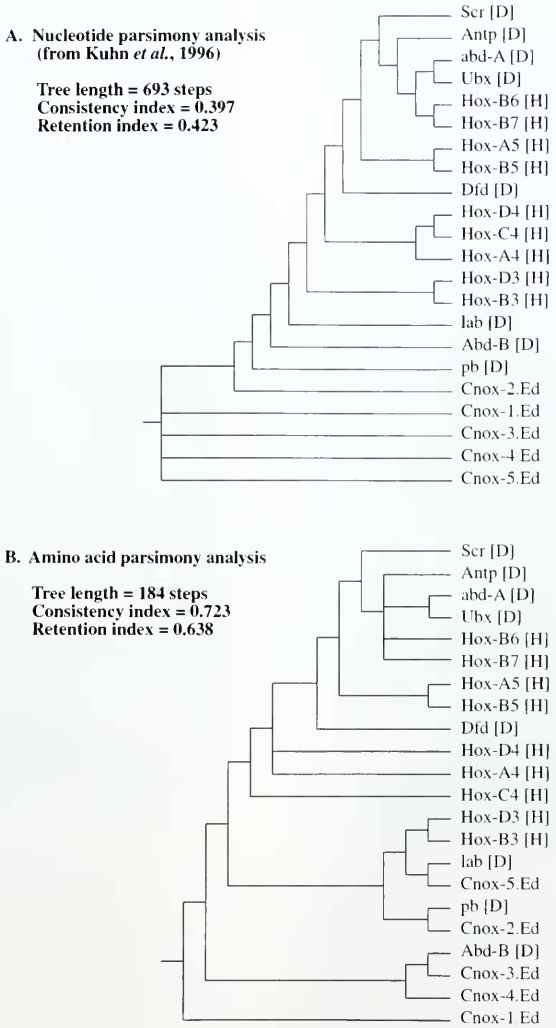

Figure 7. Comparison of parsimony analyses based on nucleotide sequences and amino acid sequences. (A) Kuhn *et al.* (1996) inferred the phylogenetic relationships of cnidarian hox gene fragments (*cnox1–cnox5* of *Eleutheria dichotoma*) to HOM/Hox homeoboxes from humans and *Drosophila* using parsimony. The tree depicted is a 50% majority-rule consensus of eight equally parsimonious trees found in a heuristic search based on unweighted nucleotide sites (180 base pairs); branch swapping by subtree-pruning-regrafting was performed on 100 random starting trees. (B) A reanalysis based on a parsimony analysis of unweighted amino acid sites (60 sites) using identical methods to search for the most parsimonious tree.

logenetic analyses suggest to us an expanded working classification of homeobox genes in the Cnidaria (Naito *et al.*, 1993) to encompass the new data from sea anemones (Table II).

The Hox class in basal metazoans

In distantly related taxa, such as vertebrates, insects, and nematodes, the genes of the Hox class are located in

evolutionarily conserved genomic clusters. Furthermore, individual Hox genes are expressed along the anterior-posterior axis of the developing embryo in the same relative order as their position in the Hox cluster: genes located towards the 3′ end of the cluster are expressed more anteriorly than genes located towards the 5′ end of the cluster. This evidence suggests that a linked cluster of Hox genes is a shared, derived metazoan character that is involved in patterning the anterior-posterior axis (Slack *et al.*, 1993; Miller and Miles, 1993). However, very little is known about the composition of the Hox cluster in basal metazoans such as the Cnidaria, or even whether such a cluster exists.

As our knowledge of Hox genes in basal metazoans increases, we should be able to establish the antiquity of the Hox cluster and of individual Hox genes, and to make inferences about the primitive function of Hox genes. Among the relevant questions are (1) How many Hox genes are found in the Cnidaria? and (2) What are the relationships of cnidarian Hox genes to Hox genes from bilaterian animals? Phylogenetic analyses of vertebrate and insect Hox genes suggest a very early trichotomy in the evolution of the Hox class—giving rise to an *Antennapedia/deformed* precursor, a *labial/proboscipedia* precursor, and an *AbdominalB* precursor (Schubert *et al.*, 1993). Previous studies revealed similarities between individual cnidarian homeoboxes and homeoboxes from the *Drosophila* genes *labial, proboscipedia, Deformed,* and *Antennapedia* (Murtha *et al.*, 1991; Schierwater *et al.*, 1991; Schummer *et al.*, 1992; Naito *et al.*, 1993). These homeobox genes have been referred to as anterior and central members of the *Drosophila* HOM/Hox cluster because their anterior borders of expression lie in the anterior half of the *Drosophila* embryo (Bartels *et al.*, 1993). A homolog of the posterior Hox genes (*AbdominalB*-like) has not been recognized in the Cnidaria.

With few exceptions (*e.g.*, Naito *et al.*, 1993), the identities of cnidarian Hox genes have been considered in isolation: Hox class genes cloned from a single species are compared to Hox sequences from *Drosophila* and vertebrates without reference to Hox genes from other cnidarians or to non-Hox homeoboxes; these comparisons have been made using pairwise alignments (*e.g.*, Schummer *et al.*, 1992; Miles and Miller, 1992; Miller and Miles, 1993) or, more rarely, phylogenetic techniques (Aerne *et al.*, 1995; Kuhn *et al.*, 1996). In this study we have attempted a more systematic approach: we used phylogenetic techniques to simultaneously assess the relationships between all the reported cnidarian homeoboxes and a broad representation of homeoboxes from bilaterian animals. As in several previous studies, our phylogenetic analyses suggest that certain cnidarian Hox genes are closely related to anterior and central members of the Hox cluster in higher metazoans (Murtha *et al.*, 1991; Schierwater *et al.*, 1991; Schummer *et al.*, 1992; Naito *et al.*, 1993). The members of cnidarian classes II, IV, V, and VI appear to be more closely related to the *labial* and *Antennapedia* homeoboxes of *Drosophila* than to the *AbdominalB* homeobox or any non-Hox homeobox. Our analyses indicate that cnidarians may possess homologs to posterior members of the complex as well as to the more anterior genes. The phylogenetic analyses (Figs. 5 and 6) suggest that members of the cnidarian homeobox classes I and Ia are most closely related to the *Drosophila Abdominal-B* homeobox, the most posterior member of the *Drosophila* Hox cluster. Therefore, distinct anterior/central (*Antennapedia/labial*-like) and posterior (*AbdominalB*-like) Hox gene lineages may have predated the split between the Cnidaria and the Bilateria (Schubert *et al.*, 1993). Additional data on cnidarian Hox genes, such as their genomic organization and axial expression, may serve to corroborate or contradict this hypothesis.

A recent paper by Kuhn *et al.* (1996) suggested a very different scenario for the origin of the HOM/Hox genes. Hox genes from triploblasts (*Drosophila* and humans) were compared with Hox genes from a cnidarian (the hydrozoan *Eleutheria dichotoma*) by using parsimony and distance methods. In the phylogenetic analyses, the resulting topologies can be rooted such that genes from triploblasts form a monophyletic group to the exclusion of all cnidarian Hox-type genes, suggesting that the common ancestor of triploblasts and cnidarians possessed only a single Hox-type gene. In other words, Kuhn *et al.* (1996) find no evidence of a Hox cluster in the common ancestor of diploblasts and triploblasts, whereas the analyses presented here are consistent with the existence of an ancestral cluster.

The difference in the two phylogenetic conclusions derives from the choice of phylogenetic characters—the analyses reported here were based on the inferred amino acid residues within the homeodomain; those of Kuhn *et al.* (1996) were based on the entire nucleotide sequence of the homeobox, including rapidly evolving, silent nucleotide sites within codons. But the relevant evolutionary time scale is very long: both cnidarian and bilaterian fossils are known from Cambrian sediments laid down more than 500 million years ago (*e.g.*, Sepkoski, 1981), so more than 1 billion years of independent evolution separate the last common ancestor of modern-day triploblasts and cnidarians. Therefore, rapidly evolving sites are likely to be saturated with phylogenetic noise. Furthermore, if there are differences in codon bias between taxa, the inclusion of silent sites in the analysis may falsely indicate phylogenetic affinity between non-homologous gene sequences derived from closely related taxa. When the phylogenetic analysis of Kuhn *et al.*, (1996) is repeated with the inferred amino acid substitu-

tions as phylogenetic characters, the result is consistent with the existence of an ancestral cluster (Fig. 7).

Confidence in inferring orthology of homeodomain sequences

In trying to infer the evolutionary relationships of homeobox genes, we are confronted by historical limitations. Our inferences are based on a small region of sequence that is highly conserved across very distantly related phyla. Sequence conservation outside the homeodomain is generally poor, so sequence alignment is difficult. The phylogenetic utility of sequences outside the homeodomain may therefore be limited. Considering this historical limitation, our inferences about the identity of several of the cnidarian homeodomains appear surprisingly robust. Several partitions on the neighbor-joining tree that suggest homology between cnidarian and bilaterian homeodomains are supported by more than 50% of bootstrap trials (for example, cnidarian *even-skipped* homologs + *Drosophila eve:* bootstrap proportion ≥ 75%). Results from computer simulations and experimental bacteriophage phylogenies suggest that bootstrap proportions over 50% can indicate a much higher than 50% probability that the corresponding branch is correct (Hillis and Bull, 1993; Felsenstein and Kishino, 1993). The results presented here agree with an emerging pattern: newly discovered homeoboxes can often be assigned to previously described classes with reasonable confidence, though the interrelationships between different classes of homeobox genes are difficult to reconstruct (*e.g.,* Finnerty *et al.,* 1996). This pattern suggests that many of the homeobox classes are extremely ancient, and that the sequences of these genes have been very strongly conserved.

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